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Diagnosis of CMT1A Duplications and HNPP Deletions by Interphase FISH: Implications for Testing in the Cytogenetics Laboratory

Lisa G. Shaffer,[1]* Gilbert M. Kennedy,[1] Aimee S. Spikes,[1] and James R. Lupski[1,2,3]

[1]*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas*
[2]*Department of Pediatrics and Human Genome Center, Baylor College of Medicine, Houston, Texas*
[3]*Texas Children's Hospital, Houston*

Charcot-Marie-Tooth (CMT) disease type 1A is an inherited peripheral neuropathy characterized by slowly progressive distal muscle wasting and weakness, decreased nerve conduction velocities, and genetic linkage to 17p12. Most (>98%) CMT1A cases are caused by a DNA duplication of a 1.5-Mb region in 17p12 containing the *PMP22* gene. The reciprocal product of the CMT1A duplication is a 1.5-Mb deletion which causes hereditary neuropathy with liability to pressure palsies (HNPP). The most informative current diagnostic testing requires pulsed-field gel electrophoresis to detect DNA rearrangement-specific junction fragments. We investigated the use of interphase FISH for the detection of duplications and deletions for these disorders in the clinical molecular cytogenetics laboratory. Established cell lines or blood specimens from 23 individuals with known molecular diagnoses and 10 controls were obtained and scored using a two-color FISH assay. At least 70% of CMT1A cells displayed three signals consistent with duplications. Using this minimum expected percentile to make a CMT1A duplication diagnosis, all patients with CMT1A showed a range of 71–92% of cells displaying at least three signals. Of the HNPP cases, 88% of cells displayed only one hybridization signal, consistent with deletions. The *PMP22* locus from normal control individuals displayed a duplication pattern in ~9% of cells, interpreted as replication of this locus. The percentage of cells showing replication was significantly lower than in those cells displaying true duplications. We conclude that FISH can be reliably used to diagnose CMT1A and HNPP in the clinical cytogenetics laboratory and to readily distinguish the DNA rearrangements associated with these disorders from individuals without duplication or deletion of the *PMP22* locus. Am. J. Med. Genet. 69:325–331, 1997.

KEY WORDS: Charcot-Marie-Tooth disease; hereditary neuropathy with liability to pressure palsies; fluorescence in situ hybridization; peripheral neuropathies; microduplication; microdeletion; prenatal diagnosis

INTRODUCTION

Charcot-Marie-Tooth (CMT) disease type 1A is an autosomal-dominant peripheral neuropathy characterized by distal muscle wasting and weakness, decreased nerve conduction velocities of usually <40 m/sec, and linkage to DNA markers in 17p12 [Lupski et al., 1991a, 1993; Kaku et al., 1993]. Most cases of CMT1A (>98%) are caused by a 1.5-Mb duplication in 17p12 [Lupski et al., 1991b; Raeymaekers et al., 1991; Pentao et al., 1992; Wise et al., 1993; Nelis et al., 1996]. This duplication includes *PMP22,* which encodes a myelin protein of the peripheral nerves whose overexpression is hypothesized to result in the CMT1A phenotype [Patel et al., 1992; Lupski et al., 1993; Patel and Lupski, 1994; Roa et al., 1993a, 1996]. Hereditary neuropathy with liability to pressure palsies (HNPP) is also an autosomal-dominant demyelinating peripheral neuropathy, although the clinical manifestations and histopathology are distinct from other neuropathies, including CMT1A [Chance et al., 1993; Lupski et al., 1993]. HNPP is associated with a 1.5-Mb deletion, the recip-

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*Correspondence to: Dr. Lisa G. Shaffer, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Room 15E, Houston, TX 77030.

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TABLE I. Possible Hybridization Patterns for CMT1A/HNPP FISH Assay

	Number of signals					
Diagnostic possibilities	1	2	3	4	5	6
Normal		X				
Normal with replication[a]			X	X		
Deletion	X					
Deletion with replication at intact locus[a]		X				
Duplication			X			
Duplication with replication at either locus[a]				X	X	X

[a]Values used to assess replication at a particular locus.

rocal recombination of the CMT1A duplication, and decreased *PMP22* gene dosage [Chance et al., 1993, 1994; Lupski et al., 1993; Patel and Lupski, 1994] in about 85% of cases [Nelis et al., 1996].

Current molecular diagnoses for CMT1A and HNPP include Southern blot analysis to detect dosage differences in CMT1A and loss of alleles in HNPP [Lupski et al., 1991b; Chance et al., 1993; Navon et al., 1995; Timmerman et al., 1996], PCR amplification of a polymorphic $(GT)_n$ repeat to detect three alleles in CMT1A or loss of alleles in HNPP [Lupski et al., 1991b; Blair et al., 1995; Navon et al., 1995; Timmerman et al., 1996], detection of novel junction fragments using a pulsed-field gel electrophoresis (PFGE) assay [Lupski et al., 1991b; Pentao et al., 1992; Raeymaekers et al., 1992; Wise et al., 1993; Chance et al., 1994; Roa et al., 1995; Timmerman et al., 1996], and fluorescence in situ hybridization (FISH) on interphase nuclei to detect duplications in CMT1A [Lupski et al., 1991b; Patel et al., 1992; Lebo et al., 1993; Liehr et al., 1995] and deletions on metaphase chromosomes in HNPP [Chance et al., 1993; Roa et al., 1993b]. Of these types of possible diagnostic tests available, only the PFGE assay for detecting junction fragments is performed routinely for diagnostic purposes in the United States [Roa et al., 1995; Lupski, 1996]. This assay detects a novel 500-kb junction fragment in CMT1A duplication patients and novel 820-kb and 770-kb junction fragments in HNPP deletion patients [Lupski et al., 1991b; Wise et al., 1993; Chance et al., 1994; Lorenzetti et al., 1995; Roa et al., 1993c, 1995]. Although PFGE is relatively labor-intensive, this assay has proven successful as a sensitive diagnostic method [Roa et al., 1995]. A relatively less labor-intensive, alternative approach to deletion and duplication diagnostics is FISH. Although FISH has been used to confirm or make a diagnosis of CMT1A [Lupski et al., 1991b; Patel et al., 1992; Lebo et al., 1993; Liehr et al., 1995] and HNPP [Chance et al., 1993; Roa et al., 1993b], we investigated the use of FISH for detection of duplications and deletions of the *PMP22* locus in the clinical molecular cytogenetics laboratory.

MATERIALS AND METHODS

Patient Samples

Established lymphoblast cell lines (n = 17) or blood specimens (n = 16) were obtained from 20 individuals with a known molecular diagnosis of either CMT1A or HNPP through PFGE, from 3 individuals with known cytogenetic duplications of 17p [Roa et al., 1996], and from 10 control individuals with no known peripheral neuropathy.

FISH

Cells were cultured according to standard cytogenetic laboratory procedures. Although the analysis was performed on interphase nuclei, cultures were exposed to colcemid according to standard procedures in order to obtain metaphase chromosomes in addition to interphase nuclei. The metaphase chromosomes were used to test hybridization efficiency and to confirm cases with apparent HNPP deletions.

Two-color FISH was performed as described [Shaffer et al., 1994; Roa et al., 1996] with the following modifications. Two cosmid contigs, one for the *PMP22* locus in 17p12 (cosmids 103B11, 132G8, and 77F4) [Roa et al., 1996], and one for the *FLI* locus mapped to the commonly deleted region in Smith-Magenis syndrome in 17p11.2 (cosmids 62F2, 70E2, and 92C10) [Chen et al., 1995; Roa et al., 1996], were used. The *FLI* contig marked the chromosome of interest and served as a positive control for hybridization efficiency. The *PMP22* cosmid contig was labelled with digoxigenin-11-dUTP by nick-translation and was detected with anti-digoxigenin conjugated to rhodamine (which fluoresces red). The *FLI* cosmid contig was labelled with biotin-16-dUTP by nick-translation and detected with avidin conjugated to fluorescein isothiocyanate (FITC) (which fluoresces green). The signals were amplified as described [Han et al., 1994; Roa et al., 1996]. For each cosmid contig, 20–30 ng/μl of DNA were used per hybridization reaction. Cells were counterstained with DAPI and viewed with a Zeiss Axiophot fluorescent microscope equipped with a triple-band-pass filter that allows one to visualize multiple colors simultaneously. Digital images were captured and stored using a Powergene 810 probe system (Perceptive Scientific Instruments, Inc., League City, TX) and printed using a Color/Monochrome Phasar II SDX printer (Tektronix, Wilsonville, OR).

Cells were scored in a blinded fashion, without knowledge of specimen origin or any diagnosis, by two independent observers. Each technologist scored the same prepared slide and counted 25 nuclei per patient sample, and submitted their results without consulting with the other technologist. Using the patient identification number, a third investigator compiled the results. In general, results were obtained from the initially prepared slide. Occasionally, hybridization on

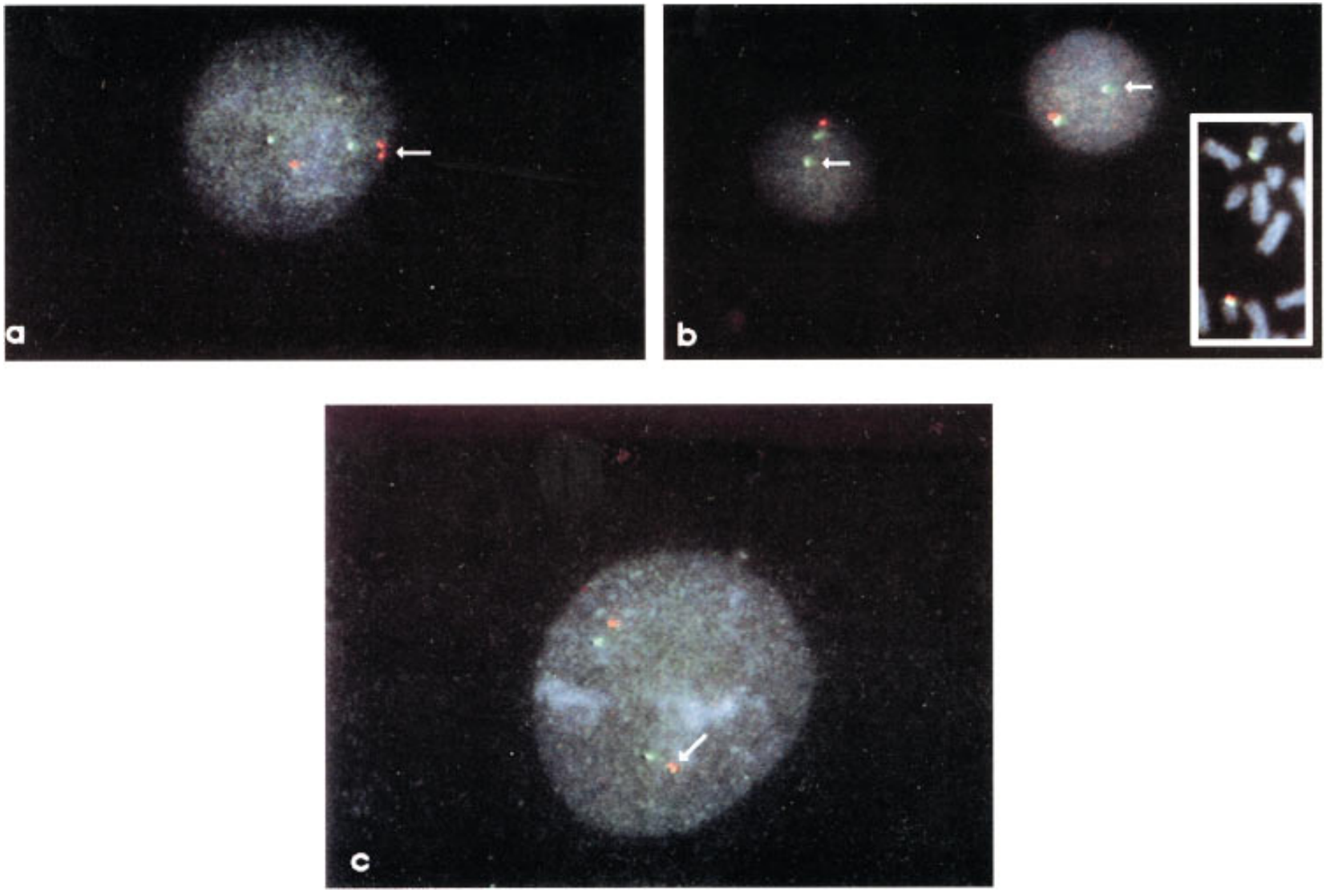

Fig. 1. Representative cells for (**a**) CMT1A showing a duplication pattern, (**b**) HNPP showing a deletion pattern in interphase nuclei and a partial metaphase spread (b, **inset**), and (**c**) a control individual showing replication at the *PMP22* locus (arrow). The *FLI* locus does not appear replicated. For each, the *PMP22* cosmid contig was labelled with digoxigenin and detected with anti-digoxigenin conjugated to rhodamine, which fluoresces red. The *FLI* cosmid contig was labelled with biotin and detected with avidin conjugated to FITC, which fluoresces green. In each interphase nucleus the normal chromosome 17 displays one green and one red signal. In cells carrying a duplication, the abnormal chromosome 17 shows one green signal and two red signals. In cells carrying a deletion, only one red signal is seen, corresponding to the normal chromosome 17. This is also illustrated on metaphase chromosomes (b, inset) where the normal chromosome 17 displays both a red and green signal, and the deleted chromosome 17 shows only the green control (*FLI*) signal.

the transformed lymphoblast samples was inadequate for scoring, and these samples were rehybridized (n = 2).

Cells were scanned using the 100× (high-power) objective. If a nucleus appeared intact and had both green and red hybridization signals, the technologist was committed to scoring that cell. The expected patterns of hybridization were scored for each nucleus. These expected patterns ranged from 1–6 signals for each locus (Table I). The percentage of cells undergoing replication at a particular locus was assessed using signals falling into the replication patterns, as indicated in Table I.

RESULTS

Thirty-three samples were investigated from peripheral blood lymphocytes and transformed lymphoblast cell lines for duplications or deletions of the *PMP22* locus, and these samples showed a range of hybridization patterns of 1–6 signals. On average, 3% of cells showed no hybridization at a specific locus. Figure 1 shows an interphase cell displaying three signals for *PMP22,* consistent with a duplication (Fig. 1a), and interphase cells showing only one *PMP22* signal, consistent with a deletion (Fig. 1b). Deletions were confirmed by examining metaphase chromosomes from the same patient sample (Fig. 1b, inset).

Using the modal number of signals (i.e., the greatest number of signals falling into a certain category), the sample could be placed into the correct diagnostic category in every case (Table II). As shown in Figure 2, the distributions of signals were significantly different between the three study populations with deletions peaking at one signal, normal controls peaking at two signals, and duplications peaking at three signals ($P < 0.0001$). The CMT1A duplication patients showed a range of 71–92% of cells displaying at least three signals (Table II). HNPP deletion patients had 80–100% of cells showing only one *PMP22* signal, consistent with hemizygosity at this locus (Table II). Controls showed 3.8% of cells displaying one signal and about 9% of cells displaying three or more signals. The remaining cells displayed only two signals, consistent with a pattern expected for normal chromosomes. Based on these findings, a minimum expected percentile for making a diagnosis was set to 70%. That is to say, at least 70% of cells must fall into one of the diagnostic categories: one signal for HNPP deletions, two signals for normal, and three signals for CMT1A duplications, in order to make a diagnosis.

Twenty cultured amniotic fluid specimens were studied to assess the replication timing in this tissue. Nineteen of the samples met the minimum criteria of 70%. Only one failed to meet this criteria, with 12% of cells displaying only one signal, 64% of cells displaying two signals, and 24% of cells displaying three or more signals. This sample result would be considered ambiguous and would warrant repeating FISH before a diagnosis could be made. This sample was probably not reflective of mosaicism, since one might expect that a true mosaic sample would show a skewing towards one signal or three signals in the abnormal cells [Sorour et al., 1995; Liehr et al., 1996].

Replication at each locus was assessed. For the *PMP22* locus, control cells and deletion patient cells were examined and showed a range of three or more signals in 0–26% of cells analyzed, with a mean of around 9% (mean ± SD = 8.8 ± 5.9%). This percentage can be interpreted as representing replication at this locus. Figure 1c shows a representative cell from a normal control individual displaying replication. In contrast, CMT duplications showed a high percentage of cells with three or more signals for the *PMP22* locus, with a mean for this population of 81.0 ± 7.7%. As can be seen in Figure 3, the number of cells showing at least three signals in true CMT1A duplication cells does not overlap with, and is significantly different from ($P < 0.0001$), those cells displaying a normal range of replication in the other study populations.

Replication was assessed at the *FLI* locus using the CMT1A, HNPP, and control samples. The *FLI* locus was found to show a range of 4–33% of cells displaying at least three signals with a mean of 15.6 ± 6.6%. As seen in Figure 4, this locus replicated relatively earlier than the *PMP22* locus, although this was not statistically significant ($\chi^2_1 = 0.39$, $P = 0.5$). Therefore, some cells may show replication for one or both *FLI* loci when *PMP22* appears replicated, but many will not (see Fig. 1c, legend). Finally, since replication timing can be tissue-specific and we wished to determine whether or not FISH could be used to test for CMT1A prenatally, the replication timing of *PMP22* was assessed in 20 cultured amniotic fluid specimens. The number of cells

TABLE II. Number of Patients Falling Into Five Diagnostic Categories for 33 Individuals Studied for CMT1A Duplications and HNPP Deletions by FISH

	Submicroscopic rearrangments		Cytogenetic duplications		Controls	
	CMT1A dup	HNPP del	*FLI* dup	*FLI/PMP22* dup	Lymphocytes/ lymphoblasts	Amniocytes
Number of patients	14	6	2[b]	1[c]	10	20
Range of signals in modal category[a]	71–92	80–100			80–96	64–100
Mean ± SD	81 ± 7.7	87.7 ± 7.1			88.2 ± 4.2	85.6 ± 9.4

[a]Percentiles are given for the *PMP22* locus. Modal signals for CMT1A, HNPP, and controls equal 3, 1, and 2, respectively. Data not shown for the *FLI* locus.
[b]Data for the *PMP22* locus have been included under the lymphocyte/lymphoblast control heading, since these cell lines have only two copies of *PMP22* because only *FLI* is duplicated by a cytogeneticly visible duplication.
[c]Data for the *PMP22* locus have been included under the CMT1A duplication heading, since this cell line has a cytogenetic duplication which includes both *FLI* and *PMP22.*

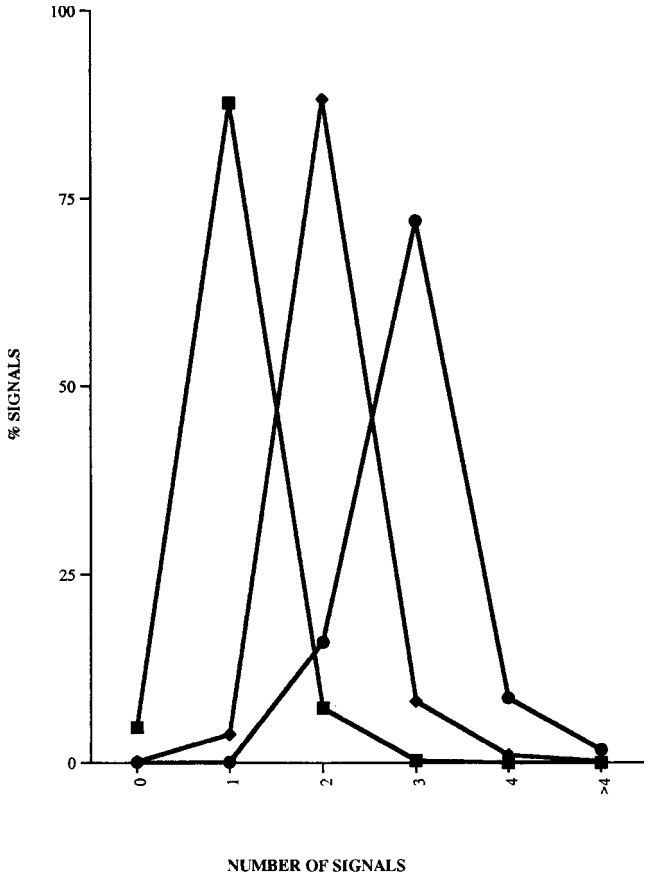

Fig. 2. Distribution of signals for the three diagnostic categories: CMT1A (●), HNPP (■), and controls (♦). Peaks correspond to the modal number of signals seen for the *PMP22* locus in each population. Data are not shown for cultured amniotic fluid specimens.

displaying at least 3 signals ranged from 0–28% with a mean of 13.6 ± 7.9%. Although slightly higher than what was found in the peripheral blood or lymphoblast cell lines, this level of replication was not significantly different from those other cell types ($\chi^2_2 = 1.82$, $P = 0.5$). This slightly higher value may reflect a somewhat different cell cycle and different replication timings in the cells that constitute amniotic fluid than those seen in lymphocytes or lymphoblasts.

DISCUSSION

FISH has been used to reliably detect microdeletions in clinical cytogenetics and, in many cases, has replaced high-resolution cytogenetic analysis [reviewed in Shaffer, 1995]. We show here that FISH can be used to reliably detect duplications in CMT1A and deletions in HNPP in the cytogenetics laboratory. All cases of duplication and deletion patients analyzed were correctly identified during this investigation. Even complex chromosomal abnormalities involving both assayed loci could be discerned. We were able to identify 3 cases with cytogenetically visible duplications of 17p using our interphase protocol, and to distinguish *FLI* duplications (n = 2) from a patient with a duplication encompassing both *FLI* and *PMP22* (n = 1).

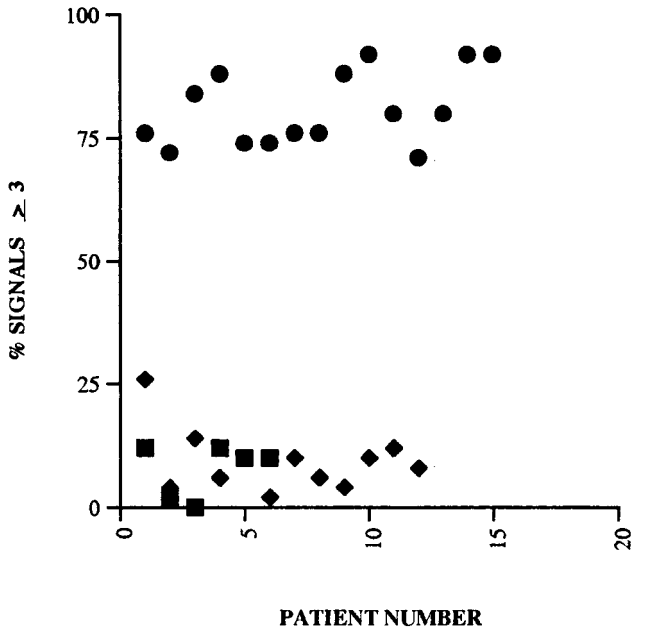

Fig. 3. Scatter plot showing percentage of cells with a duplication/replication pattern for the *PMP22* locus for each patient sample for three diagnostic categories: CMT1A (●), HNPP (■), and controls (♦). Data are not shown for cultured amniotic fluid specimens.

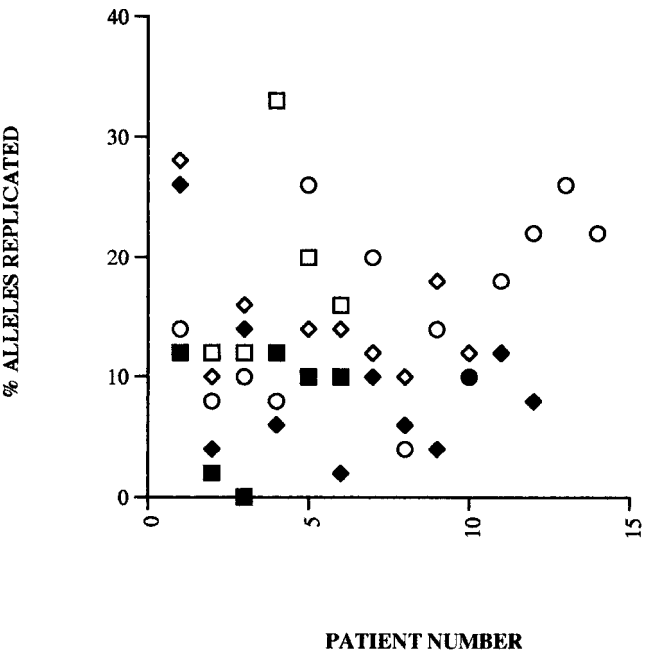

Fig. 4. Scatter plot showing percentage of cells with a duplication/replication pattern for each of the following patient sample/probe combination: CMT1A/*FLI* (○), HNPP/*PMP22* (■), HNPP/*FLI* (□), control/*PMP22* (♦), and control/FLI (◇). Although not statistically significant, the *PMP22* locus replicates relatively later as compared to the *FLI* locus. Data are not shown for cultured amniotic fluid specimens.

The predominant abnormality seen in CMT1A is a DNA duplication of a 1.5-Mb region encompassing the *PMP22* gene on chromosome 17p12. The reciprocal deletion causes HNPP. Therefore, we designed our FISH to detect the presence or absence of *PMP22,* which ac-

counts for the most common forms of CMT and HNPP, respectively. Since the respective peripheral neuropathy of CMT1A and HNPP results from segmental trisomy and haploinsufficiency of *PMP22,* smaller-sized duplications or deletions would also be expected to be detected using this assay [Valentijn et al., 1993; Palau et al., 1993; Ionasescu et al., 1993; Nelis et al., 1996]. However, very rare *PMP22* point mutations would not be detected by FISH [Valentijn et al., 1992; Roa et al., 1993b,c; Nicholson et al., 1994]. Although patients with mosaicism for CMT1A duplications have been reported [Sorour et al., 1995; Liehr et al., 1996], this assay was not designed to identify rare patients with mosaicism. Mosaic patients may result in percentiles not falling into any one category and would be considered ambiguous. Ambiguous results would warrant repeating the FISH, and if percentages still failed to meet the minimum criteria of 70%, further investigation would be required by an alternative method [Liehr et al., 1996].

One concern was the possibility of mistaking replication for duplication. *PMP22* does not appear to be expressed in lymphocytes [Patel et al., 1992] and would therefore be expected to replicate late in the S-phase of the cell cycle [Holmquist, 1987]. Although T cells comprise 55–75% of circulating lymphocytes [Barch et al., 1991], in the presence of phytohemagglutinin (PHA), only about 2–3% are stimulated to proceed to metaphase [Verma and Babu, 1989]. Therefore, the vast majority of interphase cells analyzed (T and B cells) will be in G_1 of the cell cycle and should not show a replicated allele. This observation is in general agreement with our finding of about 9% of cells displaying a replication pattern.

FISH has been used to assess the replication timing of several other loci, and has been found to be efficient and reliable [Selig et al., 1992; Kitsberg et al., 1993; Torchia et al., 1994; Gunaratne et al., 1995]. An advantage to the approach described here is that the *PMP22* locus was found to replicate relatively later as compared to the *FLI* locus (Fig. 4). This allows for *FLI* to serve not only as a control marker for chromosome 17, but also as a relative replication timing locus. Therefore, although the *PMP22* locus shows some replication (~9%), this replication pattern does not interfere with the analysis or the interpretation of samples from either the CMT1A population or nonduplicated individuals.

Allele-specific replication timing is also tissue-specific for many loci [Selig et al., 1992; Gunaratne et al., 1995]. In order to determine if our assay could reliably distinguish replication from duplication in prenatal studies, cultured amniotic fluid specimens were assayed for the replication timing of the *PMP22* locus (Table II). It was determined that the replication of this locus in amniocytes (~13%) did not differ significantly from what was found in stimulated peripheral blood lymphocytes or transformed lymphoblast cell lines. Furthermore, this level of replication was distinctly different from, and nonoverlapping with, the three signals seen in true CMT1A duplications.

In summary, FISH was shown to be a rapid, reliable, and direct approach for identifying duplications and deletions of the *PMP22* locus associated with two of the most common peripheral neuropathies, CMT1A and HNPP. FISH can therefore be used as an alternative diagnostic approach for the detection of the common molecular aberrations associated with these diseases [Lupski, 1996]. Studies of amniocytes indicate that a low level of replication at the *PMP22* locus will allow for reliable prenatal testing for these disorders. Since replication timing is tissue-specific, replication patterns must be established for chorionic villus specimens (CVS) before using CVS for diagnosing HNPP or CMT1A by FISH.

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